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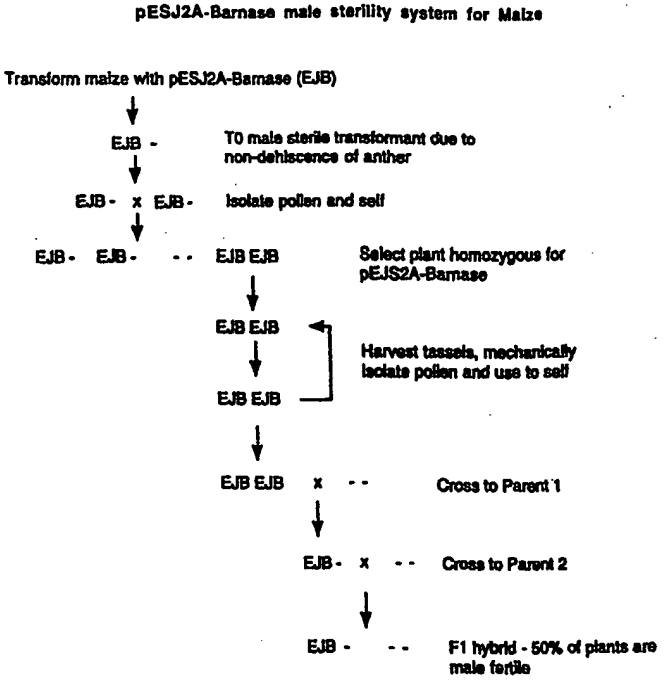
(57) Abstract

The present invention relates to the use of a nucleic acid sequence in the prevention or reduction of anther dehiscence, to transgenic plants wherein anther dehiscence is reduced or prevented and to a method for the production of such transgenic plants. Also provided is a method for producing a transgenic plant homozygous for male sterility and to a transgenic plant homozygous for male sterility.



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THE USE OF THE ARABIDOPSIS ESJ2A PROMOTER TO REDUCE ANTHER DEHISCENCE AND CREATE MALE STERILE PLANTS

The present invention relates to the use of a nucleic acid sequence in the prevention or reduction of anther dehiscence, to transgenic plants wherein anther dehiscence is reduced or prevented and to a method for the production of such transgenic plants. Also provided is a method for producing a transgenic plant homozygous for male sterility and to a transgenic plant homozygous for male sterility.

Anther dehiscence is the process whereby the anther opens to release pollen into the environment. Two processes are believed to contribute to anther dehiscence, namely splitting of the anther wall which occurs at the stomium, a specialised group of cell types running the length of the anther, and the inversion of the anther walls which exposes the pollen. Splitting of the anther wall is believed to involve cell-cell separation at the stomium, a process which is probably caused by localised expression of hydrolytic enzymes such as cellulases and polygalacturonases late in anther development. The forces required to cause anther wall inversion are probably generated by cell wall modifications of the inner anther wall. An *Arabidopsis thaliana* male sterile mutant, ms35, has been isolated in which the anthers fail to dehisce (Dawson et al (1996) J. Exp. Bot. Supplement 47: 35). In this mutant, cell separation occurs normally at the stomium but the anther walls fail to invert. This phenotype is associated with the lack of the appearance of characteristic thickenings on the inside of the anther wall. Thus, the two processes that cause anther dehiscence can be dissociated, preventing pollen release. The reduction or prevention of pollen release from plants has significant advantages. One of these is in the production of male sterile plants (useful, for example, for hybrid seed production). Another advantage is in the production of ornamental flowers that have "problem pollen", such as richly coloured pollen which is highly staining and/or difficult to remove from the skin. A further advantage of the present invention can be seen wherever the release of pollen is undesirable, for example because of its allergenic properties.

The advantages of artificial male sterile plants and the production of F1 hybrid seed are well documented. F1 hybrid seed show increased vigour, disease resistance and thus yield (WO90/08828 (Paladin/Pioneer); EP-A-0344029 (PGS); WO92/11379 (Nickerson BIOCEM)). Methods currently in use or proposed include mechanical removal of anthers, cytoplasmic or genomic male sterility and, most recently, dominant artificial male sterility (AMS) systems. Such AMS systems have numerous advantages over other systems. For example, mechanical anther removal is impracticable in most crops, cytoplasmic male sterility systems are not available in many crops and can also be associated with yield penalties and genomic male sterility is difficult to use in practice. AMS systems that have been described include the use of tapetum-specific promoters to express proteins that disrupt tapetum or pollen formation, for example Barnase (Mariani et al (1990) Nature 347: 737-741) or PR glucanase (Worrall et al (1992) Plant Cell 4: 759-771).

However, the ability to produce large numbers of male sterile (female) parents presents a number of problems in the prior art AMS systems. This is because the female cannot be maintained in a state homozygous for the AMS gene (as no viable pollen is available from the male sterile plants). At each multiplication step, the female must be crossed to an isogenic male fertile line. Only 50% of plants derived from the female will therefore carry the AMS gene and be male sterile. The male-fertile segregants must be removed at each multiplication step. This is achieved in EP-A-0344029 by linking the AMS gene to herbicide resistance and using the herbicide to remove male fertile plants. Thus, such a female multiplication system necessitates growing females and males in separate rows and removing male fertile segregants in the female rows by the use of a herbicide spray. This system is therefore wasteful in terms of area of land required for hybrid production, since pollinators are required at each step and 50% of seed from the female must be discarded at each step.

To attempt to simplify the multiplication of the female line, several ideas have been proposed. However, no successful system has yet been developed. Problems which

have been associated with prior art systems include: Those which require one mechanical cross which may not be practical for all crop species; Those which require a tightly regulated inducible system to prevent the appearance of male fertility in the hybrid production field and also require the use of chemicals which must be registered for plant use and may be difficult to apply due to climatic conditions; Those which are complex, requiring three genes and involving one mechanical crossing step; Those which require expensive seed sorting technology and where during several multiplication steps, the proportion of female seed drops.

Thus there is a continuing need to provide a practicable and economic artificial male sterility system for use in hybrid seed production. This invention describes a system based on the prevention of anther dehiscence. This system allows the female to be multiplied with the artificial male sterility gene in the homozygous state, since the female plant produces viable pollen. This system is ideally suited to crops which have high seed multiplication, large amounts of pollen, separate male and female inflorences or two or more thereof. These factors allow for easy collection of pollen from non-dehiscing anthers, facile self pollination and the minimisation of the area of plants that have to be self pollinated manually. Such an ideal crop is the monocot maize. However, the system is also applicable to most other crops, for example wheat, barley and rice.

The present invention relates to the advantages of producing plants with phenotypically normal pollen grains, within phenotypically normal anthers, but wherein the anthers do not dehisce and thus do not release the pollen grains.

According to a first aspect, the present invention provides the use of a nucleic acid sequence in the prevention or reduction of anther dehiscence in plants. The nucleic acid may be isolated or recombinant, or derived from isolated or recombinant nucleic acid. Preferably the nucleic acid is DNA.

In the present text, the meaning of "anther" are those tissues and cells of the anther which are not cells of the tapetum, pollen sac, pollen or developing pollen. The nucleic acid promoter sequence may drive expression in other parts of the plant (that is, it is not necessary that the promoter is anther-specific). However, some specificity may be advantageous, for example, where the expressed sequence is toxic to whichever cell it is expressed in. Thus, anther-specific promoters may be a preferred feature of the invention. It may also be preferable that the promoter is anther dehiscence zone specific. By anther dehiscence zone specific is meant those cells of the stomium and those cells adjacent the cells of the stomium.

The use of the nucleic acid according to the invention is in the genetic modification of a plant, a plant part or a plant cell. The use of the nucleic acid sequences, according to the invention, can result in plants with phenotypically normal pollen (fertile male gametes) and, subject only to the fact that the anther does not dehisce, phenotypically normal anthers. The production of such plants has enormous advantages.

The reduction or prevention of dehiscence according to the present invention includes the delay of dehiscence such that it achieves the desired results according to the invention. Prevention includes no dehiscence (that is no sufficient opening of the anther to release pollen). Reduction includes reduced dehiscence such that it achieves the desired result according to the present invention.

A particular group of plants to which the present invention relates are those for which a male sterility system is desired where the plant exhibits a great degree of heterosis and especially where the entire seed market consists of hybrid seed or where existing methods of hybrid seed production have not yet proven economical. Monocotyledons (monocots) are a group of plants to which the present invention relates, in particular to maize. Plants which do not posses pods or siliques are also very suitable for use according to the present invention.

The nucleic acid of the invention preferably comprises a promoter sequence which drives expression of a further nucleic acid sequence, resulting in prevention or reduction of anther dehiscence. A full or part of a nucleic acid promoter sequence which drives the desired expression may be used.

Preferably the promoter drives expression in at least the anther-dehiscence zone. It may also be preferable, as discussed above, that the promoter is anther specific or anther dehiscence zone specific (that is substantially only expressed in the anther or only in the dehiscence zone of the anther). The promoter may be one which naturally controls the expression of a cellulase or polygalacturonase in the anther. A preferred promoter is set out in Figure 3, or may be a substantially homologous sequence or a functional equivalent thereof. Suitable promoters also include the promoters of gene sequences, which gene sequences are substantially homologous to the ESJ2A gene sequence, ie the gene sequence naturally promoted or driven by the ESJ2A promoter described herein. Both promoter and coding sequence of are shown in Figure 3.

Substantially homologous sequences can be identified by the use of specialist computer programmes. According to this text a "substantially homologous sequence is one having at least 50%, up to 99% identity in steps of 10% from 50 to 90% (ie 60%, 70%, 80%, 90%). The percent identity may be determined, for example, by comparing sequence information using the (default parameters of) GAP computer program, version 6.0 described by Devereux *et al.* (*Nucl. Acids Res.* 12:387, 1984) and available from the University of Wisconsin Genetics Computer Group (UWGCG). The GAP program utilises the alignment method of Needleman and Wunsch (*J. Mol. Biol.* 48:443, 1970), as revised by Smith and Waterman (*Adv. Appl. Math* 2:482, 1981).

The promoter can be any promoter which drives expression in the anther of a plant, preferably in the anther dehiscence zone. In this text, the term "expression" means transcription, optionally followed by translation of a nucleic acid sequence.

Alternatively, the nucleic acid sequence of the first aspect of the invention comprises a sequence which when expressed, results in the prevention or reduction of dehiscence. Such a sequence is preferably under the control of a promoter which drives expression of the sequence in the anther, more preferably in the anther dehiscence zone. Further, the expression of the nucleic acid could be in any part of the plant such that it results in the prevention or reduction of anther dehiscence. Suitable nucleic acid sequences which expressed result in the prevention or reduction of anther dehiscence, include compounds which cause cell ablation, such as Diptheria Toxin (DTA), ricin, proteases, RNases, (including Barnase), lipases, endonucleases etc. Also, the nucleic acid can be one which when expressed alters the developmental fate of the anther cells, such as plant hormones. Further, the nucleic acid can be used to downregulate the genes which are required for normal anther dehiscence. Such downregulation can be achieved by expressing antisense, partial sense, full sense (transwitch) genes or by the expression of a ribozyme designed to cleave the message of such genes.

The present invention is predominantly illustrated by the use of the *Arabidopsis thaliana* promoter designated ESJ2A, although any promoter which achieves the desired result can be used. Other promoters include promoters which naturally control the expression of a cellulase or polygalacturonase enzyme in the anther, optionally in the anther dehiscence zone. For example, the ESJ2A promoter and/or coding sequence can be used to identify and isolate homologous promoters (using hybridization techniques) from other plants, in particular other plants from the *Brassica* family. Typically, the sequence, or a fraction thereof from 15 to 45 nucleic acids in length will be used to hybridize to genes from a suitable nucleic acid library, under stringent conditions. Suitable conditions may be those described in Plant Genetic Transformation and Gene Expression: A Laboratory Manual, Ed. Draper, J *et.al.* 1988, Blackwell Scientific Publications, pp 252-255, modified as follows: prehybridization, hybridization and washes at 55 to 65°C, final washes (with 0.5X SSC, 0.1% SDS) omitted.

Orthologous promoters could be identified and isolated. Orthologous nucleic acid sequences are those which are equivalent, from one species to another, in that they have the same function, but are not necessarily of identical sequence. Alternatively, suitable promoters can be obtained by isolating genes which are transcribed and/or translated in the anther, particularly in the dehiscence zone of the anther, by differential screening of plants.

A particular advantage in the present invention which is useful for the production of plants from which pollen is desired is that phenotypically normal pollen is produced. Thus, the pollen can be collected and used. Since the anthers may also be phenotypically normal, the collection of pollen therefrom may also be simple.

The present invention provides a number of significant advantages over the prior art. The present invention as an AMS system has several advantages over previously described AMS systems, particularly in the monocot maize, where the level of seed multiplication at each generation is high and where anther collection is easy. Whole male tassels can be disrupted in a blender thus allowing the pollen to be collected for pollinations. The system is genetically simple requiring only one transgene in crops where 50% fertility in the F1 hybrid crop is acceptable, such as maize (see Figure 1), or two genes when restoration is required (an appropriate restorer gene for use with ESJ2A-barnase plants would be ESJ2A-barstar). No herbicide or other selection system is required for the AMS gene. Thus there is no seed or land wastage during the female seed multiplication steps. Obtaining plants homozygous for the AMS gene is simple and during female multiplication the genotype is fixed. As for most other systems with a homozygous AMS gene, growth of female and males in rows is required only at the stage prior to hybrid production and in the hybrid production field. This extra pre-hybrid female multiplication step reduces the female multiplication area required that has to be manually pollinated. For maize, calculations suggest that the area required for the last manual pollination step is 30 ha (Greenland (1997) PBI

bulletin p 11). This would lead to the production of sufficient F1 hybrid seed to supply the entire USA market of 30,000,000 ha. Thus, the effort required in pollen isolation and pollination at these early female multiplication steps is not excessive, especially since it is not critical to remove all the male tassels from the females.

A further application of the control of anther dehiscence relates to advantages in the production of ornamental flowers. Plants such as lilies, orchids and tulips can produce large attractive anthers which may contain richly coloured pollen. On anther dehiscence, coloured pollen causes a serious problem since it is highly staining and difficult to remove from skin, clothing and other items. For this reason anthers are mechanically removed from the majority of Liliaceae (and some other flowers) before they are sold. As well as being a laborious process, the removal of the anthers makes the flowers significantly less visually attractive. Since the Liliaceae are largely vegetatively propagated, it would be feasible to select for male sterile mutants or introduce a tapetally expressed AMS gene such as A9-Barnase (WO 92/11379). However, these plants would usually contain aborted pollen grains or no pollen grains thus reducing the colouration of the anthers and consequently the attractive appearance of the flowers. Additionally, early pollen abortion may cause the anther to shrivel and late pollen abortion will not prevent anther dehiscence. It has also been reported in some species that male sterility will affect flower size and colouration. The present invention overcomes these problems since completely normal pollen grains can be formed within phenotypically normal anthers. The only difference is that the anthers will not dehisce releasing the staining pollen. Transformants according to the present invention are thus both more economic to produce and would command a higher price since they are more visually attractive.

A distinct and further advantage is the use of the present invention to prevent or reduce anther dehiscence wherever the release of pollen is undesirable, such as because of its allergenic properties. The present invention is thus useful in the

avoidance or reduction of pollen allergens and may be extremely effective in the control of asthma (in particular severe asthma) caused by pollen release.

In the first aspect of present invention where the nucleic acid sequence comprises a promoter, the promoter is preferably operatively linked to a nucleic acid sequence (as described above) which when transcribed, optionally followed by translation, reduces and/or prevents anther dehiscence. This can be achieved by a variety of ways. The promoter can control the expression of a compound which causes cell ablation such as Diptheria toxin (DTA), ricin, proteases, RNases, (including Barnase) lipases, endonucleases etc. Also, the promoter can be used to express genes that alter the developmental fate of the anther cells such as plant hormones or used to specifically downregulate genes which are required for normal anther dehiscence. Such downregulation can be achieved by expressing antisense, partial sense, full sense (transwitch) genes or by the expression of ribozymes designed to cleave the message of such genes. The choice of promoter/expressed sequence combination is important to ensure the normal functioning of cells (as far as required) except for the DZ cells of anther.

The present invention is exemplified by the linkage of the *A.thaliana* ESJ2A promoter to the RNase Barnase and transformation of this construct into *B.napus*, resulting in a proportion of transgenic plants that are male sterile due to prevention of anther dehiscence. Examination of these plants showed that the anther stomium cells have been killed, preventing the expression of hydrolytic enzymes that are required for cell-cell separation in the stomium. Viable pollen was mechanically isolated from the non-dehisced anthers and used in self and cross pollinations. Thus it was possible to obtain plants homozygous for the ESJ2A-Barnase gene. This enabled the use of the ESJ2A promoter in a simple but extremely effective male sterility system. The initial male sterile transformant was selfed using pollen isolated mechanically from the non-dehiscing anthers. Homozygous progeny were identified and subsequent multiplication of this female line was achieved - again by selfing with mechanically

isolated pollen. When enough female seed was obtained it was sown in rows next to a male pollinator (Parent 1). Seed from the females was heterozygous for the dominant male sterile gene and was used again in a further cross to a male pollinator (Parent 2) thus producing the desired F1 hybrid seed from Parents 1 and 2 (see Figure 1).

According to a second aspect of the invention there is provided a transgenic plant wherein anther dehiscence has been reduced or prevented. The transgenic plants are those, in particular which do not have pods or siliques. Where the plant does have pods or siliques, such as plants of the family Cruciferae or the family Leguminosae, the availability of reduced pod shatter as well as reduced anther dehiscence can be borne in mind. Preferably the plant is a monocot, such as maize, wheat, a fodder grass, a banana tree, a palm, an orchid, a tulip or a lily (member of the family Liliaceae) and/or melon, cucumber, tomato, pepper and willow.

Such a plant preferably has, introduced into its nucleic acid, a construct which comprises a promoter. The promoter drives expression in the anther operatively linked to a nucleic acid sequence which, when expressed reduces and/or prevents anther dehiscence.

Methods for introducing nucleic acid sequences into the genome of a plant are well known in the art. Suitable systems for monocots and dicots are known. For example, reference is made to transformation of maize by particle bombardment described in Gordon-Kamm *et al.*, 1990, Plant Cell, vol. 2, pp 603-617, transformation of maize by Agrobacterium in Ishida *et al.*, 1996, Nature Biotechnology, vol 14, pp 745-750, and transformation of wheat by particle bombardment in Weeks *et al.*, 1993, Plant Physiology, vol 102, pp 1077-1084.

Alternately, the plant has introduced into its nucleic acid, a construct which comprises a nucleic acid sequence which when expressed, results in the prevention or reduction of anther dehiscence. Such a sequence should be introduced into the nucleic acid of

the plant such that it is under the control of a promoter which drives expression of the nucleic acid in the anther, preferably in the anther dehiscence zone (more preferably expression specific to these regions of the plant).

Most preferably the plant has introduced into its nucleic acid a construct which comprises a promoter as described above according to the second aspect of the invention, operably linked to a nucleic acid which expressed results in the prevention or reduction of anther dehiscence (also as described according to the second aspect of the invention).

All preferred features of the first aspect of the invention also apply to the second.

The plant may have itself been transformed or may have been derived (directly or indirectly) from such a plant. The nucleic acid may be endogenous, i.e. from the same plant or a plant of the same species, or exogenous, i.e. from an alternative species. The nucleic acid used to transform plants is preferably isolated or recombinant and is preferably DNA. Also covered by the invention is propagating material derived from a plant according to the second aspect of the invention, particularly seed material.

A third aspect of the invention relates to a method for producing a plant according to the second aspect, comprising transforming a plant, plant part or plant cell with a nucleic acid construct which comprises either a promoter which drives expression of a nucleic acid sequence to cause the prevention or reduction of anther dehiscence, or a nucleic acid which when expressed causes the prevention or reduction or plant dehiscence or a construct which comprises the promoter operably linked to the nucleic acid sequence. The steps of transforming the plant are well known in the art and any can be used according to the present invention.

All preferred aspects of the first and second aspect also apply to the third.

Propagating material derivable from the second aspect (and produced by the third aspect) is also provided.

A further aspect of the invention relates to a method for producing a plant homozygous for male sterility, the method comprising the production of a plant heterozygous for male sterility where normal pollen production is obtained, using the pollen obtained to self fertilize the plant or to fertilize another plant at least heterozygous for the same allele, and obtaining homozygous propagating material, capable of producing a plant homozygous for male sterility. A transgenic plant homozygous for male sterility

The present invention is illustrated using the figures, in which:

Figure 1 shows an outline of the use of the control of anther dehiscence in hybrid seed production in maize.

Figure 2 is a schematic diagram of the *A.thaliana* genomic clones that hybridise to the *B.napus* cDNA. The EcoRI-BamHI hybridising fragment cloned into pTZ18R is shown.

Figure 3 shows the DNA sequence of the *A.thaliana* ESJ2A promoter, the coding sequence for the ESJ2A gene and the predicted amino acid sequence encoded.. The primers used to PCR promoter fragments are indicated (PGL is the primer used to PCR the 'long' promoter (pPGL) and PGS is the primer used to PCR the 'short' promoter (pPGS)).

Figure 4 shows the construction of fusions of the pPGL and pPGS promoters to GUS.

Figure 5 shows the histochemical staining for GUS expression in pods and anthers from *B.napus* transformants. A) Developmental appearance of GUS activity in the

dehiscence zone (DZ) of developing pods from transformant WP271-7-9. Numbers in the figure below the pods indicate age of the pod in 'days after anthesis' (DAA). B) Section of a pod DZ from transformant WP271-1-14. DZ cells that express GUS were red under the filter used (appear in the figure as dark areas). C) GUS expression in the anther DZ (stomium) of transformant WP271-7-9. D) Section of an anther DZ from transformant WP271-7-14. Cells which expressed GUS were red (some seen here indicated by lines) and can be seen next to where cell separation has occurred at the stomium.

Figure 6 shows the construction of fusions of the pPGL and pPGS promoters to Barnase.

Figure 7 shows sections of a wild-type (A) anther and an anther from a pPGS-Barnase transformant (WP273-6-9) (B). Both anthers are of similar ages. The wild-type anther has dehisced, whereas the pPGS anther has not. Consequently this PGS-Barnase plant is male-sterile, but can be seen to produce pollen.

The present invention is illustrated by the following non-limited examples.

EXAMPLES.

1) Isolation of the ESJ2A promoter.

The isolation of the *B.napus* SAC66 cDNA is described in Jenkins et al., (1996) J. Exp. Bot. 47, 111-115 and in WO96/30529. The *B.napus* cDNA was used to screen an *A.thaliana* genomic library (Paul et al., (1992) 19, 611-622) in order to isolate the *A.thaliana* orthologous gene. The genomic inserts from three hybridising phage were analysed further to localise the region hybridising to the *B.napus* cDNA probe (Figure 2, shows a diagrammatic representation of the extent of the region from a Lambda clone 2A which hybridises to the *B.napus* cDNA probe). A 7kb EcoRI, BamHI

fragment encompassing the hybridising region was cloned from Lambda 2A between the EcoRI and BamHI of pTZ18 (Figure 2) and partially sequenced to determine the orientation of the ESJ2A promoter. The DNA sequence of the ESJ2A promoter is shown in Figure 3. The predicted coding regions of SAC66 and ESJ2A are 87% identical at the DNA level and 87% identical at the protein level. To clone the putative promoter from *A. thaliana* in a form in which it could be easily linked to Barnase and other genes the following oligonucleotides were designed:-

5' TGATCTAGAGACATCCACGAAAACG	3'	PGL
5' TTCTCTAGACGTGTTGTACTAAC 3'	PGS	
5' GGGCCATGGCGATTTTGATCGG 3'	2A Nco	

Primers PGL and PGS are engineered to contain an XbaI site and 2A NcoI an NcoI site. The positions at which these primers bind is indicated on Figure 3. PCR using the *Tli* proof-reading thermostable polymerase (Promega) thus generated a 1.9 kb promoter fragment using primers PLG and 2ANco (PGL promoter) and a 1.4kb promoter fragment when using primers PLS and 2ANco (PGS promoter). These fragments were cloned into SmaI-cut pTZ18R forming pPGL and pPGS (Pharmacia) and sequenced.

2) Linkage of promoter fragments to GUS and analysis of transgenic *B.napus* plants.

The PGS and PGL promoters were first cloned as SstI-NcoI fragments from pPGS and pPGL between the SstI and NcoI sites of pDH68 forming pWP271 and pWP272 respectively (Figure 4). pDH68 consists of a Pea Plastocyanin promoter (Pwee and Gray (1993) Plant J. 3, 437-449) linked to an intron GUS gene (D. Twell, Leicester University) cloned between the SstI and EcoRI sites of the vector pJIT30 (Guerineau et al., (1990) Plant Mol. Biol. 15, 127-136). Thus the Pea Plastocyanin promoter is replaced with the PGL or PGS promoters. The pPGS-GUSint-CaMV polyA or pPGL-GUSint-CaMV polyA chimeric genes are cloned as XbaI, XhoI fragments between the

XbaI and SalI sites of the binary vector pSCV nos-nptII (W0 96/30529), forming pWP271-SCV and pWP272-SCV, and transformed into *B.napus* (var. Westar) by agrobacterial transformation essentially as described in Moloney M et al., (1989) Plant Cell Reports 8, 238-242.

Figure 5 shows pods and anthers from transformed plants histochemically stained using X-Gluc (5-bromo-4-chloro-3-indolyl-ß-D-glucuronide) for GUS activity. In WP271-SCV and in WP272-SCV plants, GUS activity is found in the DZ of the pod in the anther DZ and in the seed funiculus. In some plants, the level of expression in the pod DZ is higher than in the anther DZ whereas in other plants the reverse is observed. The reason for the variability in the relative expression levels of GUS in between the anther and pod DZ is unknown. It could be due to the influence of flanking plant sequences (positional effect) or be due to differential methylation of areas of the promoter that are responsible for either expression in the anther DZ or pod DZ. This variability in promoter expression also indicates that it will be possible to obtain modified ESJ2A promoters which are either entirely anther-specific or entirely pod-specific. This can be achieved by deleting or mutating elements in the promoter sequence which are required for pod-specific expression or anther specific expression respectively.

3) Linkage of promoter fragments to Barnase and analysis of transgenic *B.napus* plants.

The PGS and PGL promoters were first cloned as SstI-NcoI fragments from pPGS and pPGL between the SstI and NcoI sites of pWP128 (Paul et al., (1992) Plant Mol. Biol. 19, 611-622) forming pWP273 and pWP274 respectively (Figure 6). The chimeric genes are then cloned as XbaI, XhoI fragments into XbaI, SalI - cut pSCV nos nptII forming pWP273-SCV and pWP274-SCV and transformed into *B.napus*. The plants showed a range of pod shatter resistance due ablation of the pod DZ. The majority of plants 6 out of 6 WP273-SCV and 16 out of 19 WP274-SCV plants) were also male

sterile due to ablation of the anther DZ and the consequent prevention of anther dehiscence. Some plants were however pod shatter resistant but male fertile. Figure 7 shows a section of a wild-type anther that has dehisced and an anther from a PGS-Barnase plant that has failed to dehisce. Such pPGS-Barnase and pPGL-Barnase plants are consequently male sterile but produce pollen (Figure 8B).

4) Production of male-sterile maize plants.

The pESJ2A-Barnase constructs pWP273 and pWP274 were digested with XbaI and XhoI and used to independently transform maize using a particle bombardment technique. The majority of transformants exhibited male sterility due to a lack of anther dehiscence. These plants exhibited no additional phenotype. Pollen was isolated from non-dehiscent anthers and used to self the male sterile plants. The selfed progeny contained individuals that were homozygous for the transgenes.

5) Production of Lily plants with non-dehiscent anthers.

The pESJ2A-Barnase constructs pWP273 and pWP274 were digested with XbaI and XhoI and used to independently transform lily using a particle bombardment technique. The majority of transformants exhibited male sterility due to a lack of anther dehiscence. These plants exhibited no additional phenotype and had anthers that were of normal size and colour. Pollen extracted from the non-dehiscent anthers had normal colouration and was viable.

CLAIMS

1. Use of a nucleic acid sequence in the prevention or reduction of anther dehiscence.

2. Use, as claimed in claim 1, in the genetic modification of a plant.

3. The use as claimed in claim 1 or claim 2, wherein the nucleic acid sequence comprises a promoter sequence, which drives expression of a further nucleic acid sequence which results in the prevention or reduction of anther dehiscence.

4. The use as claimed in claim 3, wherein the promoter drives expression in the anther dehiscence zone.

5. The use, as claimed in claim 1 or claim 2, wherein the nucleic acid sequence comprises a sequence which when expressed, results in the prevention or reduction of anther dehiscence.

6. The use, as claimed in claim 5, wherein the nucleic acid sequence is expressed in the anther dehiscence zone.

7. The use, as claimed in anyone of claims 1 to 6, in a plant which does not have pods or siliques.

8. The use as claimed in claim 7, wherein the plant is maize.

9. The use as claimed in any one of claims 1 to 8, wherein the nucleic acid sequence comprises a promoter which naturally controls the expression of a cellulase or polygalacturonase enzyme in the anther.

10. The use as claimed in any one of claims 1 to 9, wherein the nucleic acid sequence comprises a promoter which comprises one or more regulatory elements from the region 5' to the coding region (as set out in Figure 3).

11. The use as claimed in any one of claims 1 to 10, wherein the cause of anther dehiscence reduction or prevention is cell ablation.

12. The use, as claimed in claim 11, wherein the reduction or prevention of dehiscence is caused by the expression of Barnase.

13. The use as claimed in any one of claims 1 to 12, wherein the nucleic acid, which when expressed, alters the developmental fate of a cell in which it is expressed.

14. The use as claimed in claim 13, wherein the nucleic acid which is expressed encodes a plant hormone.

15. The use as claimed in any one of claims 1 to 4, wherein the cause of anther dehiscence reduction or prevention is the downregulation of genes required for normal anther dehiscence.

16. A transgenic plant, especially a transgenic maize plant wherein anther dehiscence is reduced or prevented.

17. A plant as claimed in claim 16 which has, introduced into its nucleic acid a construct comprising a promoter operably linked to a sequence, wherein the

promoter drives expression of the sequence to which it is operably linked, in the anther resulting in reduces or prevents anther dehiscence.

18. A plant as claimed in claim 16 or claim 17, wherein the promoter drives expression in the dehiscence zone of the anther.

19. A plant as claimed in any one of claims 16 to 18 wherein the promoter naturally controls expression of a polygalacturonase enzyme in the anther.

20. A plant, as claimed in claim 17, which has introduced into its nucleic acid, under the control of a promoter which drives expression in the anther, a nucleic acid sequence, the expression of which reduces or prevents anther dehiscence.

21. A plant as claimed in any one of claims 16 to 21 wherein the cause of anther dehiscence reduction or prevention is cell ablation.

22. A plant as claimed in claim 21 wherein the cell ablation is caused by expression of Barnase.

23. A plant as claimed in anyone of claims 16 to 22, wherein expression of the nucleic acid reducing and/preventing anther dehiscence alters the developmental fate of a cell.

24. A plant as claimed in claimed 23, wherein the expression causing reduction or prevention of anther dehiscence is of a plant hormone.

25. A plant as claimed in any one of claims 16 to 25, wherein the cause of anther dehiscence reduction or is the downregulation of genes required for normal anther dehiscence prevention.

26. A plant as claimed in any one of claims 16 to 25 which is maize.

27. A method for producing a transgenic plant as claimed in any one of claims 16 to 26 comprising the introduction into a plant, plant part or plant cell, of a nucleic acid construct, the intorudction of which produces a promoter which drives expression in the anther operably linked to a nucleic acid sequence which, when expressed, reduces or prevents anther dehiscence.

28. Propagating material derived from a plant as claimed in any one of claims 16 to 27.

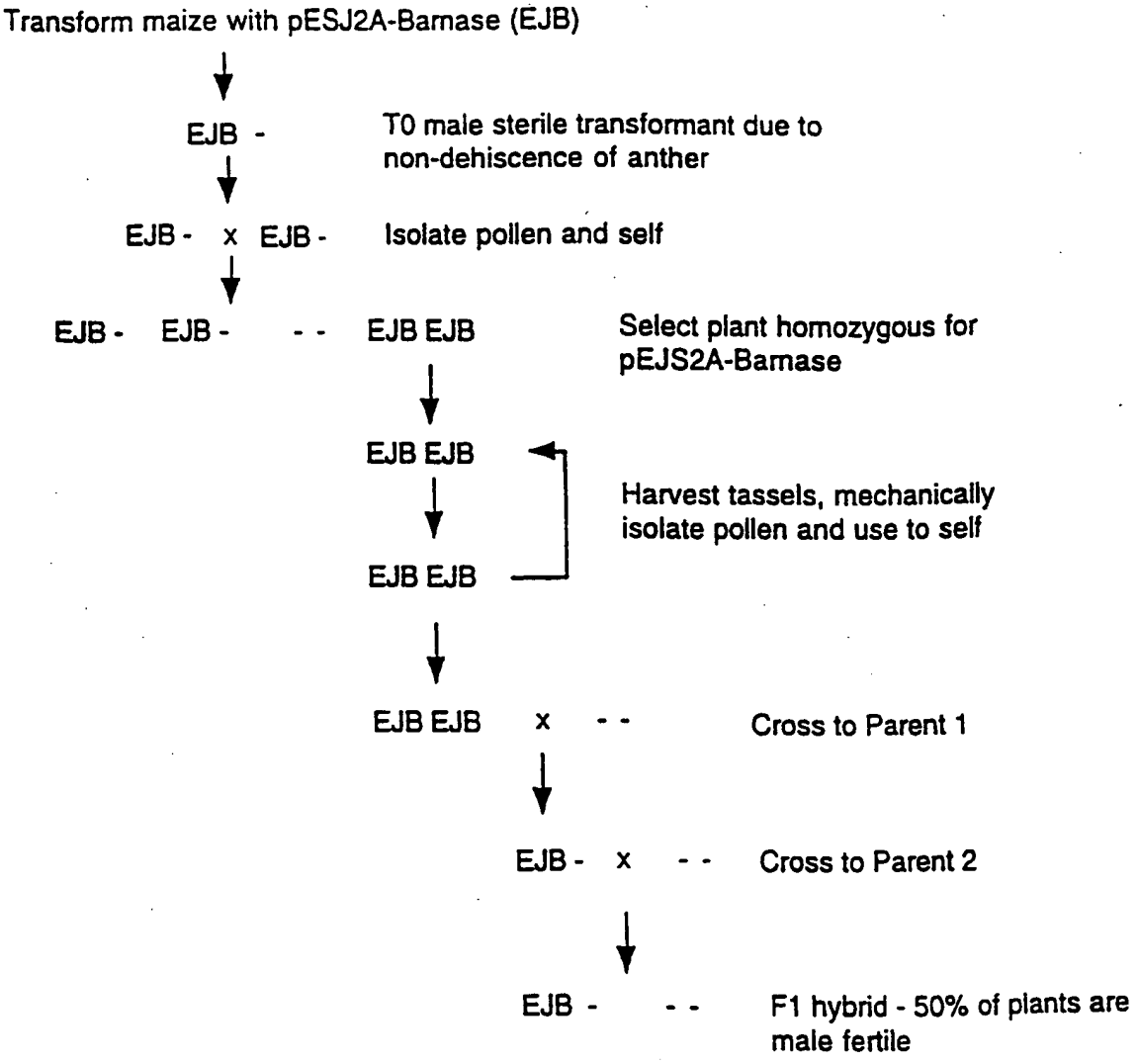
29. Propagating material as claimed in claim 28 which is a seed.

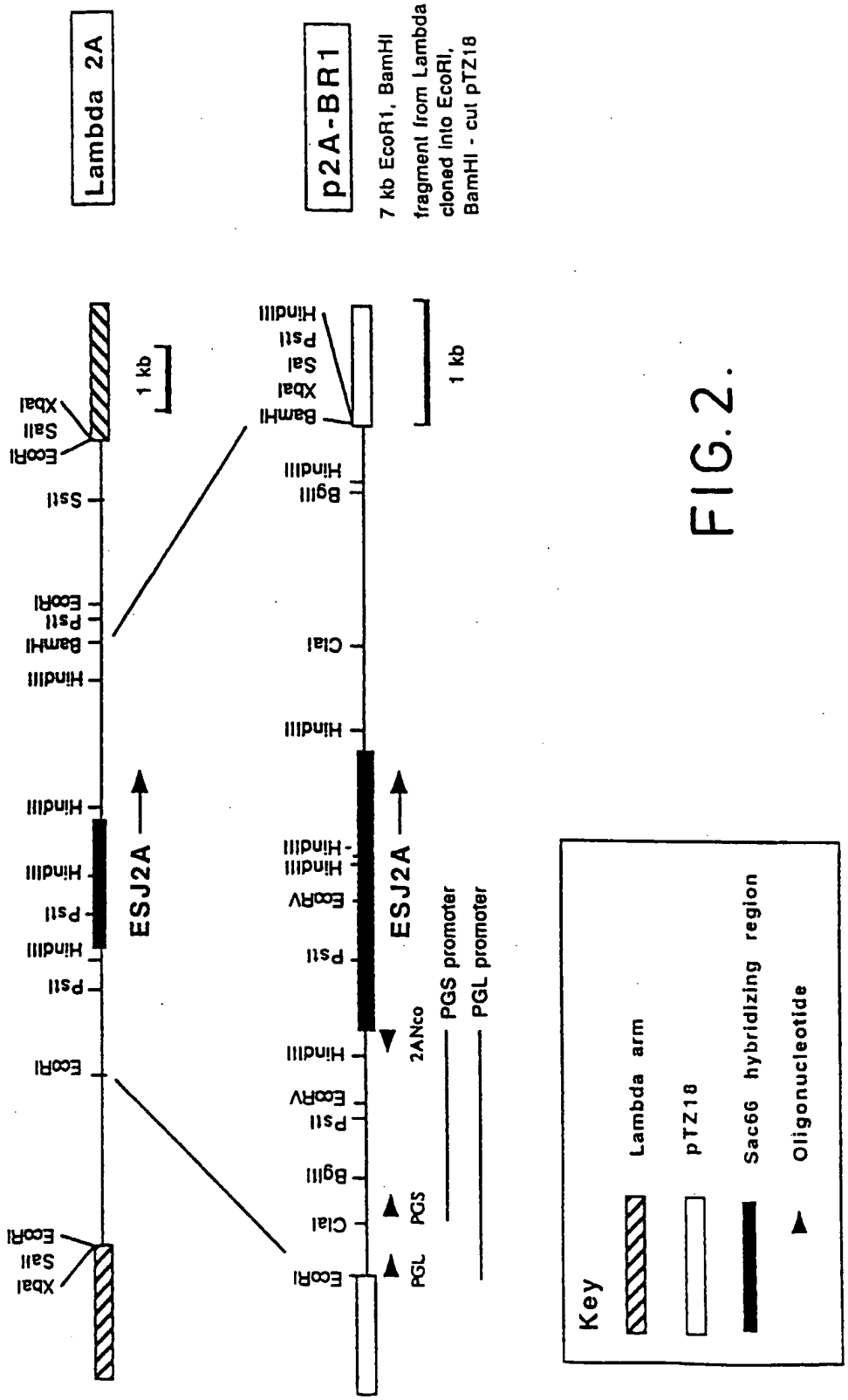
30. A method for producing a transgenic plant homozygous for male sterility, the method comprising the production of a plant heterozygous for male sterility wherein normal pollen production is obtained, using the pollen to self fertilize the plant, or to fertilize another plant heterozygous for the same allele and obtaining homozygous propagating material, capable of producing a plant homozygous for male sterility.

31. A transgenic plant homozygous for male sterility.

FIG. 1.

pESJ2A-Barnase male sterility system for Maize

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1 kb
1 kb
XbaI
SalI
EcoRI
SstII
EcoRI
PstI
BamHI
HindIII
HindIII
PstI
HindIII
HindIII
EcoRI
XbaI
SalI
EcoRI
ESJ2A
ESJ2A
HindIII
BamHI
XbaI
SalI
PstI
HindIII
BglII
HindIII
ClaI
HindIII
HindIII
EcoRV
PstI
HindIII
EcoRV
PstI
BglII
ClaI
EcoRI
2ANco
PGS
PGL
PGS promoter
PGL promoter
Key
Lambda arm
pTZ18
Sac66 hybridizing region
Oligonucleotide

FIG. 2.

FIG. 3.

GTCTTTTCCATCTTTTTCAGTGTTTAAGTTTATCTTTCTTTGCTCTTTTACCTACACCAAGTAGTAATATTTTAAAAA 78

TGAAATAGTGAAATAGTGACATCCACGAAAACGATGTGCAAGTTGAATCGTAACCATTAATTAAGAAAAATACAAGAG 156
TGATCTAGAGACATCCACGAAAACG-> PGL-prom

ATAAATTCAAAGAAGGAAGCAATAACTAAGGCCAAGTTATAACAATGACTAGATAAATTTCCAAAGACATGACTCTCG 234

ACCATACCTTTAGGTCTCTCAAGCAAACTACGCACATGCCTTTAACTTCTGCCTATTTTTACACCATACGCGCATCTA 312

CATATTAACACTAGTATATACAAATATTTGTATTAATTAATTGGTAATTACAAAACAATTAGATAAAGTATAATAAGG 390

ATAAGTACGTGATCTTTTGTGCTTATTAATCGATAGGAATATATGTATGGGCATCAAGTCTTCACCATTGTTCATGAT 468

GTCCCATGCATTGTAGTATTTTTGTAGGTTTCTTTAAGCCGCAACCCGGTTGGCCGCTAGTTTCTTTGAAATTATACT 546

AAAAGACACCTTTGAAATCATAGAAAACAATGCTTGACATGGTTTCAATAACAAAATACTAAACTTTAAATAATTGAC 624

ATATTAGTAAATCATAAGATTCATAACTAAATTTCCAAAGTAACTTGGTATTTTTATTTTTATTTTGGAAGAAAATTG 702

GTATTTTCATTATGATAAATTAATGTATGGTTTTAATTAACTAATTAATGAACCATATATACCAAATTTCACGAGACG 780
TTCTCTAGACG

TGTTGTACTAACTACTAAAGACTTTTGAGACTAATATTTTTCATCATTTGGGAGTTGAATGCAAAGTTGTTCCCGATA 858
TGTTGTACTAAC-> PGS prom

AACTTTTCAGTTGATTTTCAAGCAAATTAGATCTTGTTGGCGTTATTATTTACCATACCATTTCAATCATTACATTAG 936

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CAATTTAATTTCGGTATACCAATTCATGTTGGTTTAATGATTAGTAATTTATTAGAGGATGGAAATCAGGTGTCAAAC 1248

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AAATGTTACTGTGAATAGTATATGATGATGAAAATTAACTCTTTTTCTAATTTACAGAAAAAAATACGTAACTAATTA 1872

AACTGTGAAACTCTCAATGTAAAATTAAAACAATTAAAAAAAAAAATAGGGGGGAGAGGAGGTAAGGCTTGAAGCTTAA 1950

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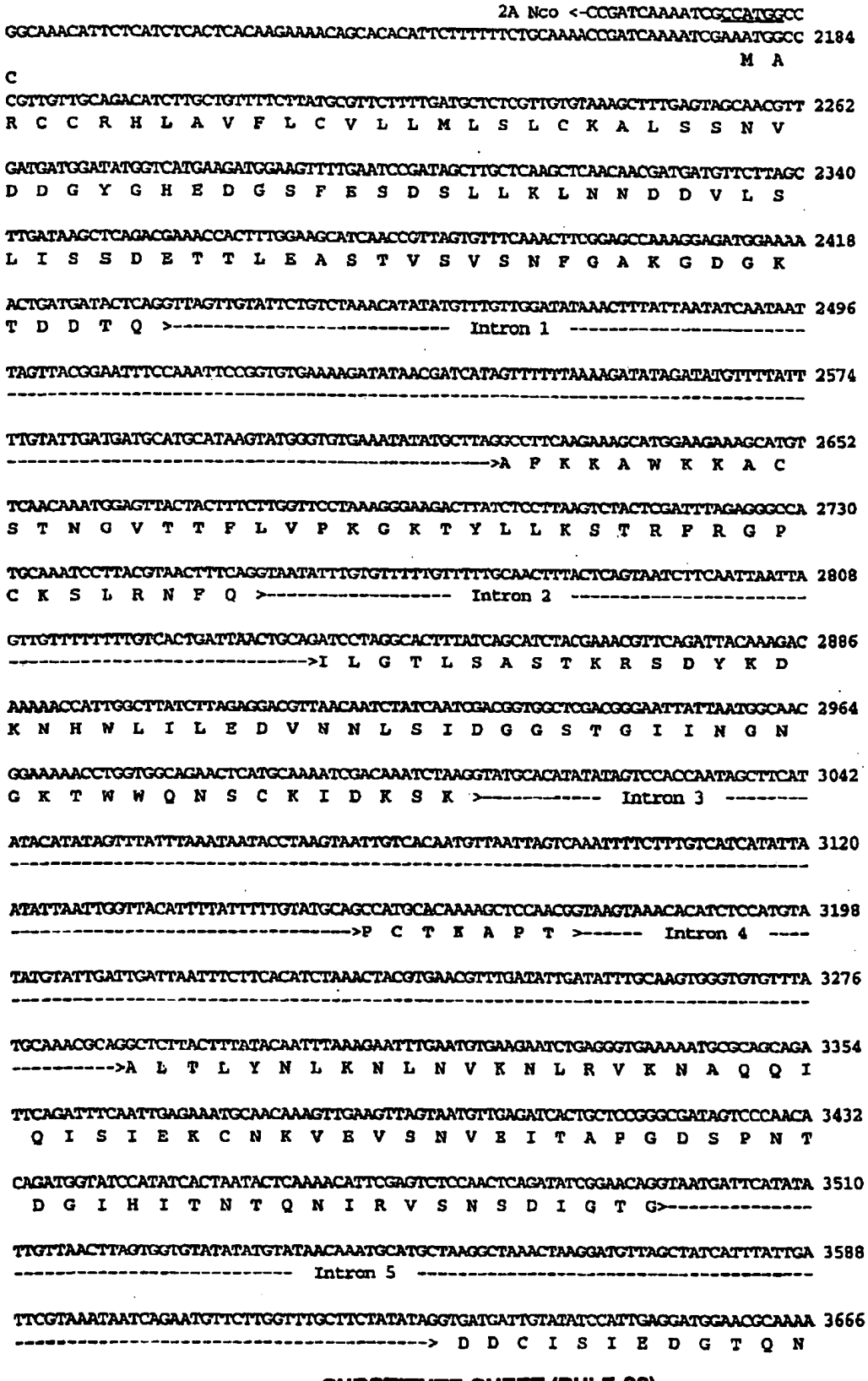
FIG.3 CONT'D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L I S S D E T T L E A S T V S V S N F G A K G D G K
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S T N G V T T F L V P K G K T Y L L K S T R F R G P
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K N H W L I L E D V N N L S I D G G S T G I I N G N
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D G I H I T N T Q N I R V S N S D I G T G>----------
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----------> D D C I S I E D G T Q N

FIG.3 CONT'D

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-------------------------------------------------> I  G  S  L  G  D  D  N  S

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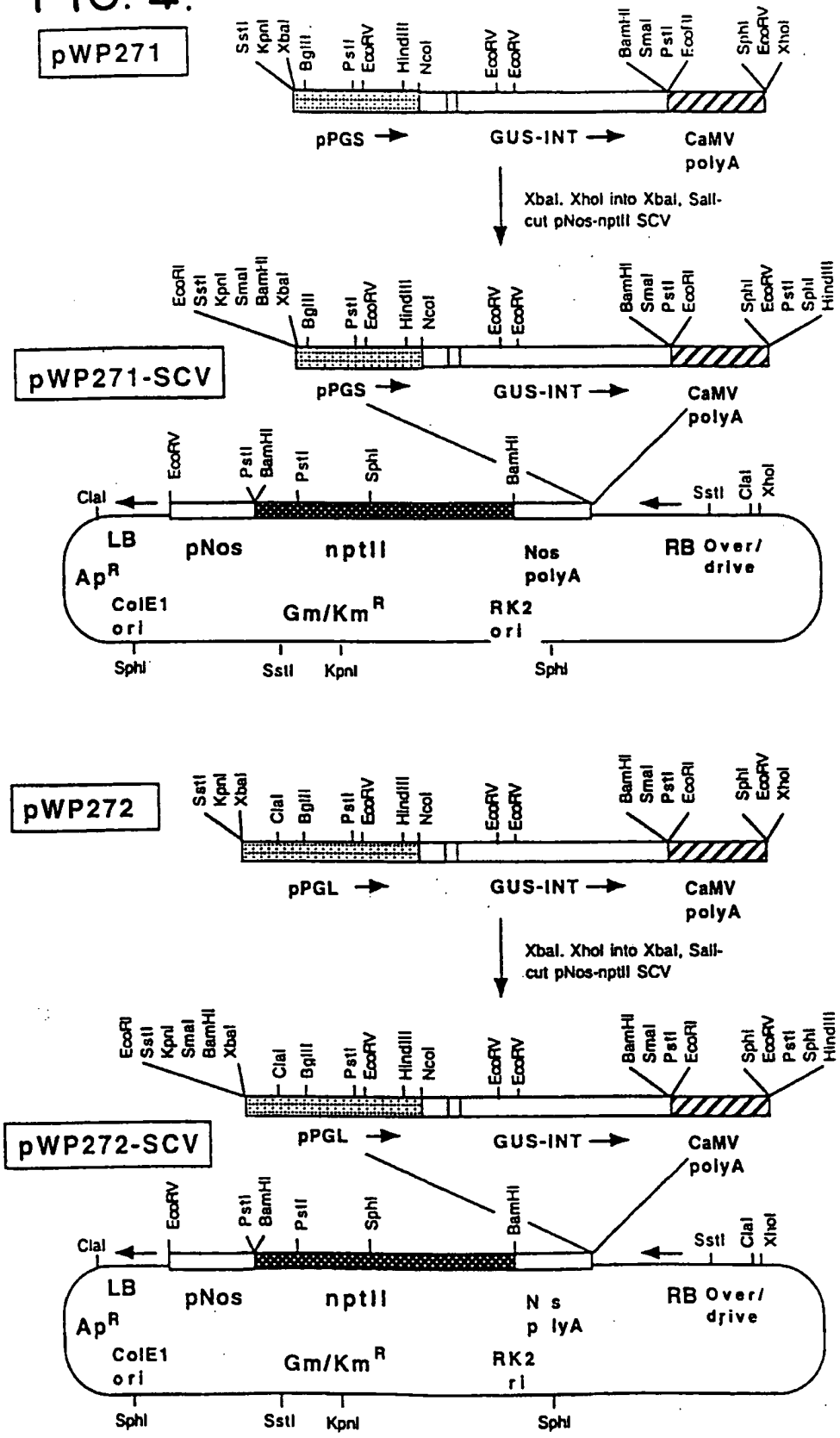
FIG. 4.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SstI KpnI XbaI BglII PstI EcoRV HindIII NcoI EcoRV EcoRV BamHI SmaI PstI EcoRI SphI EcoRV XhoI
pPGS
GUS-INT
CaMV polyA
XbaI, XhoI into XbaI, SalI-cut pNos-nptII SCV
pWP271-SCV
EcoRI SstI KpnI SmaI BamHI XbaI BglII PstI EcoRV HindIII NcoI EcoRV EcoRV BamHI SmaI PstI EcoRI SphI EcoRV PstI SphI HindIII
pPGS
GUS-INT
CaMV polyA
EcoRV PstI BamHI PstI SphI BamHI
ClaI
SstI ClaI XhoI
LB
pNos
nptII
Nos polyA
RB Over/drive
ApR
ColE1 ori
Gm/KmR
RK2 ori
SphI
SstI KpnI
SphI
pWP272
SstI KpnI XbaI ClaI BglII PstI EcoRV HindIII NcoI EcoRV EcoRV BamHI SmaI PstI EcoRI SphI EcoRV XhoI
pPGL
GUS-INT
CaMV polyA
XbaI, XhoI into XbaI, SalI-cut pNos-nptII SCV
pWP272-SCV
EcoRI SstI KpnI SmaI BamHI XbaI ClaI BglII PstI EcoRV HindIII NcoI EcoRV EcoRV BamHI SmaI PstI EcoRI SphI EcoRV PstI SphI HindIII
pPGL
GUS-INT
CaMV polyA
EcoRV PstI BamHI PstI SphI BamHI
ClaI
SstI ClaI XhoI
LB
pNos
nptII
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RK2 ri
SphI
SstI KpnI
SphI

FIG. 5.

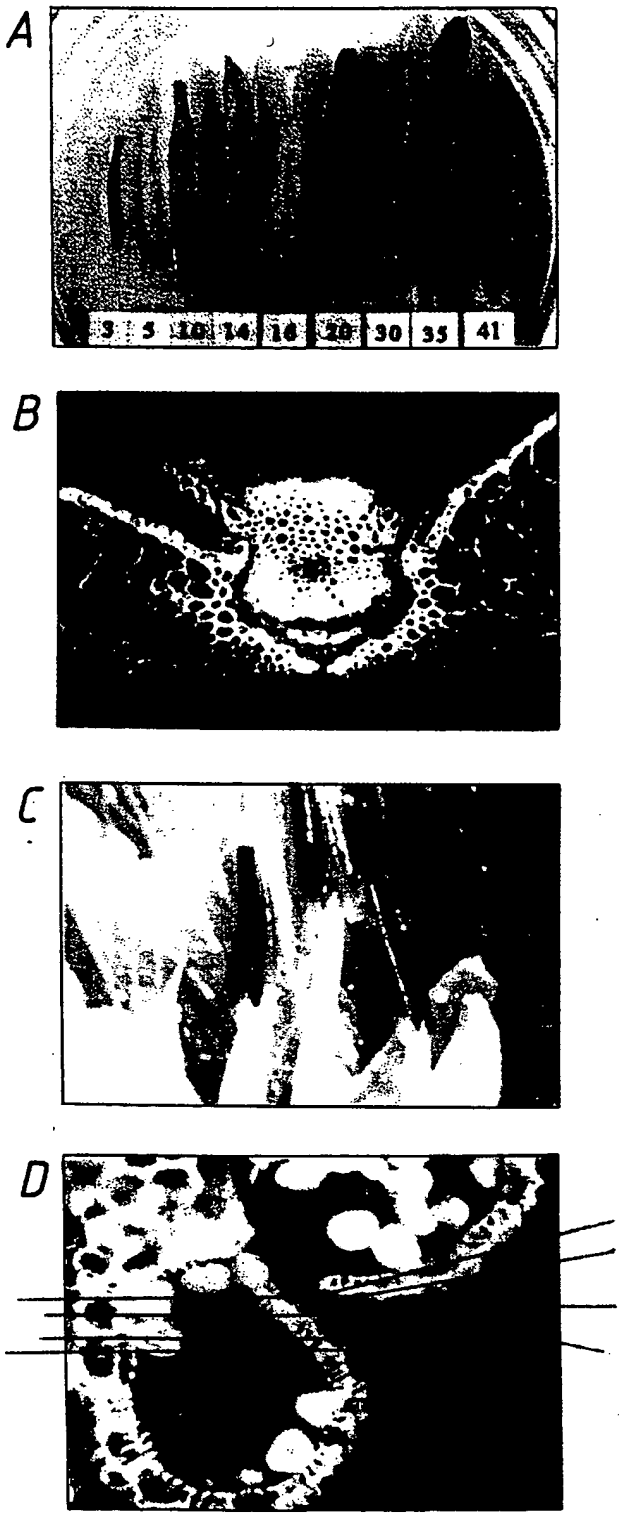


FIG. 6.

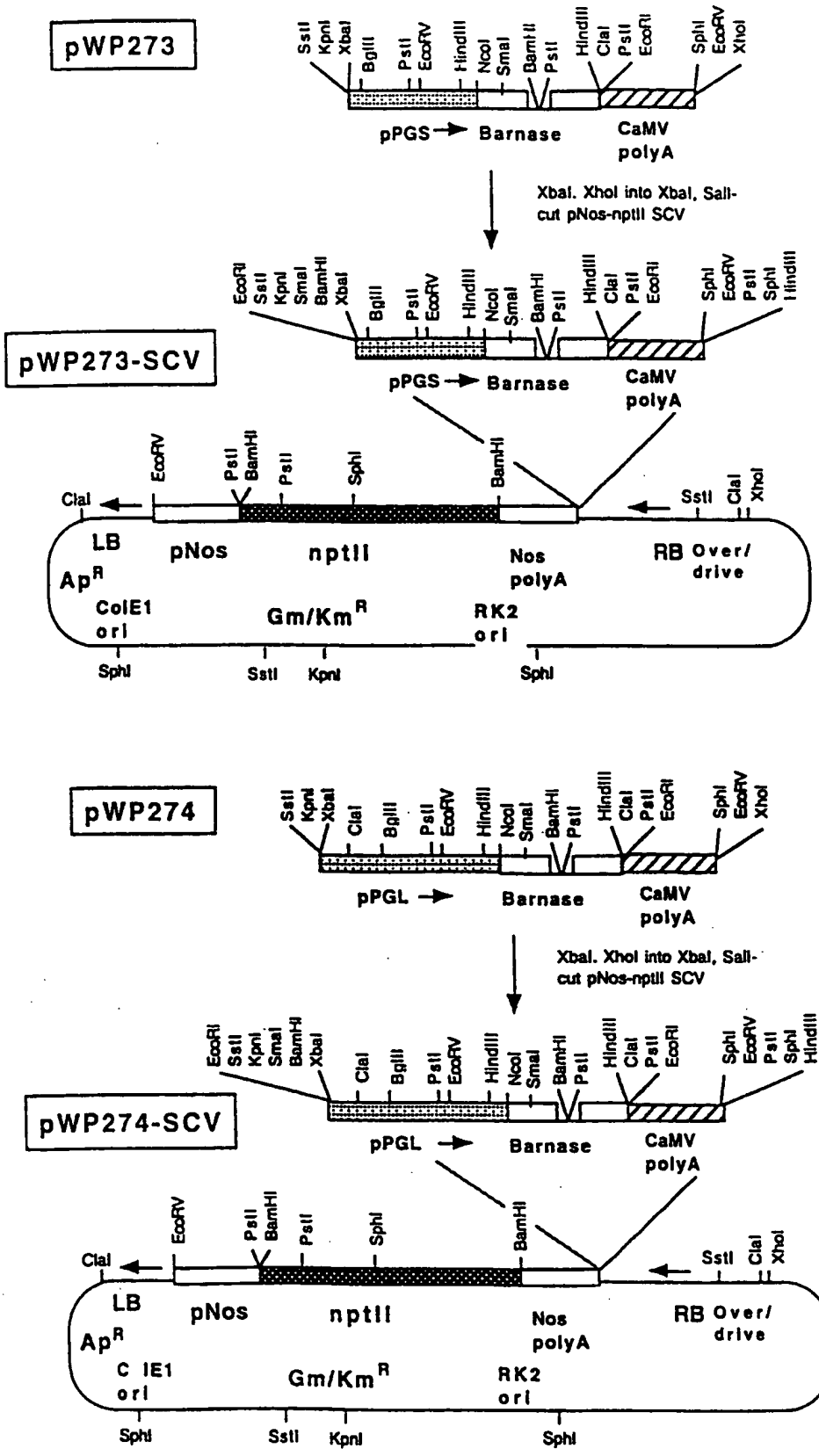
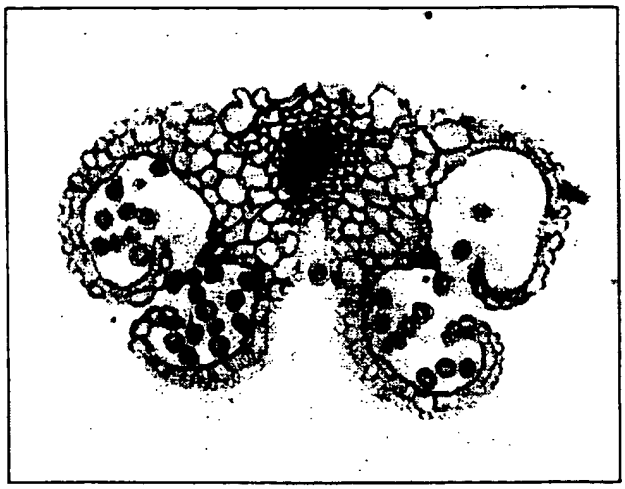

pWP273
SstI KpnI XbaI BglII PstI EcoRV HindIII NcoI SmaI BamHI PstI HindIII ClaI PstI EcoRI SphI EcoRV XhoI
pPGS → Barnase
CaMV polyA
XbaI. XhoI into XbaI, SalI-cut pNos-nptII SCV
pWP273-SCV
EcoRI SstI KpnI SmaI BamHI XbaI BglII PstI EcoRV HindIII NcoI SmaI BamHI PstI HindIII ClaI PstI EcoRI SphI EcoRV PstI SphI HindIII
pPGS → Barnase
CaMV polyA
EcoRV PstI BamHI PstI SphI BamHI
ClaI
SstI ClaI XhoI
LB
pNos
nptII
Nos polyA
RB Over/ drive
ApR
ColE1 ori
Gm/KmR
RK2 ori
SphI SstI KpnI SphI
pWP274
SstI KpnI XbaI ClaI BglII PstI EcoRV HindIII NcoI SmaI BamHI PstI HindIII ClaI PstI EcoRI SphI EcoRV XhoI
pPGL → Barnase
CaMV polyA
XbaI. XhoI into XbaI, SalI-cut pNos-nptII SCV
pWP274-SCV
EcoRI SstI KpnI SmaI BamHI XbaI ClaI BglII PstI EcoRV HindIII NcoI SmaI BamHI PstI HindIII ClaI PstI EcoRI SphI EcoRV PstI SphI HindIII
pPGL → Barnase
CaMV polyA
EcoRV PstI BamHI PstI SphI BamHI
ClaI
SstI ClaI XhoI
LB
pNos
nptII
Nos polyA
RB Over/ drive
ApR
C IE1 ori
Gm/KmR
RK2 ori
SphI SstI KpnI SphI

FIG. 7.

A



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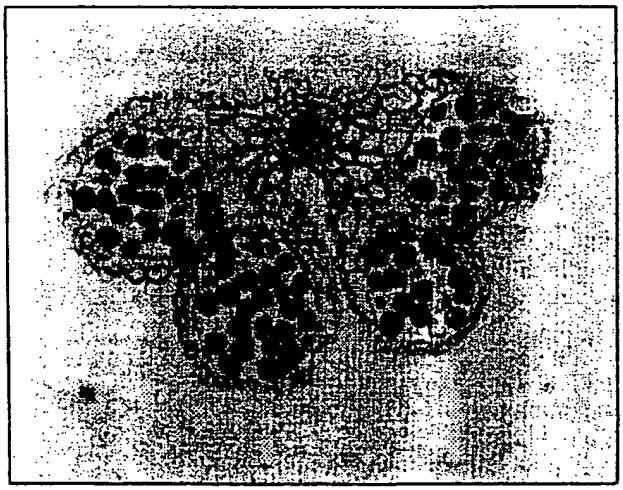

INTERNATIONAL SEARCH REPORT

Interr al Application No
PCT/GB 98/02752

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12N15/56 C12N15/82 A01H5/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C12N A01H

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	EP 0 344 029 A (PLANT GENETIC SYSTEMS NV) 29 November 1989 cited in the application see the whole document, esp. example 10	1-3,5, 11,13, 16,17, 20,21, 27-29
X	THORSNESS M. ET AL.: "Genetic ablation of floral cells in Arabidopsis" THE PLANT CELL, vol. 5, no. 3, 1993, pages 253-261, XP002092038 see the whole document	1-3,5, 11,13, 16,17, 20,21, 23,27-29

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[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

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"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
3 February 1999	26/02/1999

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Kania, T

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INTERNATIONAL SEARCH REPORT

Intern ıal Application No
PCT/GB 98/02752

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

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X	BEALS T. AND GOLDBERG R.: "A novel cell ablation strategy blocks tobacco anther dehiscence" THE PLANT CELL, vol. 9, no. 9, September 1997, pages 1527-1545, XP002092039 see esp. p. 1535 ff.	1-6, 11-13, 16-18, 20-23, 27-29
X	GOLDBERG R. ET AL.: "A novel cell-ablation strategy for studying plant development" PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY OF LONDON, SERIES B, BIOLOGICAL SCIENCES, vol. 350, 1995, pages 5-17, XP002092040 see page 9 ff.	1-6, 11-13, 16-18, 20-23, 27-29
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A	GOLDBERG R. ET AL.: "Anther development: Basic principles and practical applications" THE PLANT CELL, vol. 5, no. 10, - 1993 pages 1217-1229, XP002092041 see page 1222 - page 1227	1-31

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Information on patent family members

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